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A role for nonapeptides and dopamine in nest-building behaviour

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ABSTRACT

During nest building in zebra finches (*Taeniopygia guttata*), several regions in the social behaviour network and the dopaminergic reward system, two neural circuits involved in social behaviour, appear to be active in male and female nest-building finches. As the nonapeptides mesotocin and vasotocin, and the neurotransmitter dopamine, play important roles in avian social behaviour, we tested the hypothesis that mesotocinergetic-vasotocinergetic and dopaminergic neuronal populations in the social behaviour network and dopaminergic reward system, respectively, are active during nest building. We combined immunohistochemistry for Fos (an indirect marker of neuronal activity) and arginine vasotocin, mesotocin, or tyrosine hydroxylase on brain tissue from nest-building and non-nest-building male and female zebra finches and compared Fos immunoreactivity in these neuronal populations with the variation in nest-building behaviour. Fos immunoreactivity in all three types of neuronal populations increased with some aspect of nest building: (1) higher immunoreactivity in a mesotocinergetic neuronal population of nest-building finches compared to controls; (2) increased immunoreactivity in the vasotocinergetic neuronal populations the more material picked up by nest-building males and the more time a male spent in the nest with his mate, and (3) increased immunoreactivity in a dopaminergic neuronal population the longer a male nest-building finch spent in the nest with his mate. Together these findings provide evidence for a role of the mesotocinergetic-vasotocinergetic and dopaminergic systems in avian nest building.

Introduction

Understanding the neurobiology of reproductive behaviour in vertebrates has long been a focus of neuroendocrine research (e.g. [1]). In birds, these studies have typically investigated the production and

perception of courtship song [2], affiliation [3], copulation [4], and parental care [5]. One key avian reproductive behaviour that has received much less attention is nest-building behaviour.

The current consensus is that two evolutionarily conserved neural circuits, the social behaviour network and dopaminergic reward system, are important for most reproductive behaviour in vertebrates [6]. Functionally, the social behaviour network is thought to be involved in the production of courtship, sexual, affiliative, and aggressive behaviours [7] whereas the dopaminergic reward system is implicated in the motivation to perform these behaviours [8]. Recent studies from our laboratories have revealed that neuronal activity increases in brain regions within both the social behaviour network and dopaminergic reward system the more nest-building behaviour male and female zebra finches exhibit [9].

Many of the brain regions in the social behaviour network and dopaminergic reward system that exhibit elevated neuronal activity with nest-building behaviour are known to contain populations of neurones characterised as using specific signalling molecules to transmit neuronal information to target brain regions [6]. In zebra finches, these populations include the vasotocinergic and mesotocinergic parvocellular neuronal populations in the medial bed nucleus of the stria terminalis (BSTm) of the social behaviour network, which release the nonapeptide hormones vasotocin (the avian analogue of arginine vasopressin in mammals) and mesotocin (the avian analogue of oxytocin in mammals), respectively. In addition to releasing these nonapeptides, which may bind to receptors in brain regions including the social behaviour network [10], these neuronal populations also innervate hypothalamic and social behaviour network targets including the medial preoptic area [10], which exhibits elevated neuronal activity during nest building [9]. These populations are distinct from the parvocellular paraventricular neurones that form part of the hypothalamic- pituitary-adrenal stress response axis that are

vasotocinergic [11]. Central effects on behaviour via dendritic release from magnocellular vasotocin and mesotocin neuronal populations may be predicted to occur in birds as reported in mammals [12-14] but this remains to be established, and was not the focus of this current study. In the dopaminergic reward system, dopaminergic neuronal populations in the ventral tegmental area and central gray synthesise and release the neurotransmitter dopamine, which may act on dopaminergic receptors in both the striatum and regions in the social behaviour network including BSTm and the septum [15,16], amongst other sites.

The actions of the vasotocinergic, mesotocinergic, and dopaminergic neuronal populations appear to mediate many reproductive behavioural functions that are enhanced during the breeding season. For example, in zebra finches, vasotocinergic neurones in BSTm are thought to be involved in affiliative behaviour and courtship [17], and dopaminergic neurones in the central gray appear to be involved in vocal communication with conspecifics [18]. Nonapeptidergic signalling has diverse behavioural effects in mammals: oxytocin can suppress appetite; it stimulates female sexual receptivity and male sexual arousal, grooming behaviour, and is anti-anxiolytic [19] and vasopressin has been implicated in social behaviour including pair bonding social recognition and aggression [20]. These nonapeptides also have a well-established important role in parental behaviour in mammals [19,21-23]: for example, *i.c.v.* oxytocin infusions induce maternal behaviour in rats and mice [23,24] and oxytocin signalling appears to be involved in nest-building behaviour in rodents [25,26]. Therefore, we hypothesized that the vasotocinergic, mesotocinergic, and dopaminergic neuronal populations within the social behaviour network and dopaminergic reward system are also involved in nest building, a key parental behaviour in birds. In this study, we compared nest-building behaviour in male and female zebra finches with concurrent neuronal activity, as measured indirectly by the number of neurones producing the immediate early gene protein product Fos, in vasotocinergic and mesotocinergic neuronal populations in subdivisions of BSTm and dopaminergic neuronal populations in the ventral tegmental

area and central gray. As we previously established that neuronal activity in the dorsal subdivision of BSTm (BSTmd) increased the longer female finches spent in the nest [9] and Klatt and Goodson found that the delivery of a mesotocin antagonist in the zebra finch brain reduces the amount of time nest-building female finches spent in the nest [28], we hypothesised that mesotocinergetic neurones in the ventral subdivision of BSTm (BSTmv) may be involved in the amount of time a female finch spends within the nest and predicted that Fos and mesotocin neurone immunoreactive colocalisation will reflect this brain-behaviour relationship. Neuronal activity also increases in BSTmd during nest building [9] so we also predicted that Fos immunoreactivity in vasotocinergetic and mesotocinergetic neurones in BSTmd would be higher in nest-building finches compared to controls.

Finally we sought to further understand the role of the ventral tegmental area in nest building [9]. If increases in neuronal activity involve the dopaminergic neuronal population in this region, then Fos immunoreactivity within dopaminergic neurones in the ventral tegmental area should increase the more often male finches pick up nest material.

Materials and methods

Animals

We bred thirty-two adult zebra finches (n = 16 male, n = 16 female) in captivity at the University of St. Andrews, St. Andrews, Scotland, UK. Prior to experimentation, we housed all birds in single-sex group cages containing 10-20 birds and *ad libitum* access to finch seed mix and water. All birds were maintained on a 14L:10D light:dark cycle (lights on at 08:00) between 19-27°C and 50-70% humidity. All procedures in this study were performed with permission from the University of St. Andrews Animal Welfare and the Ethics Committee and the UK Home Office (PPL. 60/3666).

Treatment group assignment

We randomly paired zebra finches in opposite-sex pairs in wooden/wire mesh cages (40 x 30 x 39 cm) and housed finch pairs within the same room as the single-sex group cages. Pair cages were fitted with a wooden nest cup (11 x 13 x 12 m), the floor of the cage was covered with bedding chips, and finch pairs were given access to finch seed mix and water *ad libitum*. Finches were paired for a minimum of one week before receiving 15 cm lengths of string (No. 4 Polished Cotton Twine; Rope Source, UK) as nest material. Prior to receiving string, finch pairs regularly filled their nest cups with bedding chips from the cage floor and some females laid eggs in these bedding nests. We removed bedding and eggs from nest cups daily.

After at least a week following pairing, we gave four pairs of birds 50 pieces of string at 12:00. We inspected cages 24 hours later to identify pairs that had begun building a string nest in their nest cup. To create an experimental cohort, we randomly assigned each finch pair that had begun building a nest to one of the two behavioural treatment groups (nest-building and control group). We selected only finch pairs that had begun building a nest to ensure that all pairs included in this study were motivated and capable of building nests prior to nest building observation.

After allocating birds to treatment groups, we removed all string from the cages of both finch pairs in the experimental cohort and also removed the nest cup from the cage of the control finch pair. We removed the bedding chips from the cages of both pairs to prevent the birds using them for nest building, lined the cage floors with black plastic, and moved both pairs to the experimental room. We repeated this selection procedure until we had eight nest-building and eight control zebra finch pairs.

Nest building

Once in the experimental room, the control and nest-building pairs were visually but not acoustically isolated from each other by a wooden barrier. To record out-of-nest cup behaviour, we positioned a camcorder in front of each pair's cage (Sony Handycam AVCHD, Model no. HDR-CX115E) and to record in-nest cup behaviour we suspended a bird-box camera inside each pair's cage (SpyCameraCCTV, Bristol, UK). We left each cohort undisturbed in the experimental room for 24 hours to habituate.

Thirty minutes after lights on, the morning following the habituation, we gave the nest-building pair 250 pieces of string and began filming both pairs. An experimenter observed the birds from outside the test room via a window until the male of the nest-building pair made three consecutive trips with nest material from the cage floor to the nest. We recorded these trips as the time at which the male began to build and sacrificed the birds 90 minutes later. If the male began building immediately after receiving material, we delayed the start of the observation for 15 minutes to avoid sampling Fos production in the brain associated with the bird seeing the experimenter.

Behaviour coding

We encoded the birds' behaviour using Noldus Observer (TrackSys Ltd., Nottingham, U.K.) behavioural analysis software and measured the occurrence of behaviours performed 80-50 minutes prior to sacrifice, a time bin in which Fos production is associated with these nest-building behaviours [9]. Briefly, we measured instances of *hopping*, *feeding*, *drinking*, *preening*, *scratching*, and *allopreening* in all birds. In males, we recorded the number of *song bouts* and the *time spent singing (s)*. In nest-building birds, we measured six nest-building behaviours: *pick up* (when the bird picked up a piece of

string), *put down* (when the bird deposited a piece of string into the nest), *tuck* (when the bird used its beak to push a piece of string into the nest while in the nest cup), *nest visits*, and *time in nest (s)*. We also measured *time together (s)* in the nest (the duration both members of a nest-building pair spent together in the nest cup).

Tissue collection

After 90 minutes following the start of nest building, an experimenter entered the room to confirm visually that string was deposited in the nest cup. Once confirmed, we terminally anaesthetised (0.2 ml i.p.; Dolethal) both pairs of birds and rapidly dissected their brains from their skulls. We fixed brains via submersion in 4% paraformaldehyde in phosphate-buffered saline (0.1M; pH = 7.4) for six days and then moved the brains into in 20% sucrose in phosphate-buffered saline overnight and then in 30% sucrose in phosphate-buffered saline for another night to cryoprotect them. An experimenter removed cerebella from the rest of the brains and froze both the cerebella and remaining brain on pulverised dry ice and stored all neural tissue at -80°C before transporting the brains on dry ice to the Roslin Institute, University of Edinburgh, Roslin, UK where the samples were again stored at -80°C. An experimenter sectioned brains coronally (section thickness = 52 µm) using a freezing microtome and collected sections in four, alternating series in cryoprotectant and stored the sections at -20°C until free-floating immunohistochemical processing. The cerebella were processed for Fos immunoreactivity in a separate study (Hall et al., in prep).

Double-label immunohistochemistry

Three series of sections were rinsed four times in 0.2% Triton X-100 (Sigma) in 0.1M phosphate buffer (PBT) and once in 0.1M phosphate buffer before being incubated in 0.3% H₂O₂ in phosphate

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buffer for 15 minutes at room temperature to reduce endogenous peroxidase activity. Following three PBT rinses, sections were incubated in 10% Normal Goat Serum (Vector Laboratories) in PBT for 60 minutes at room temperature. Sections were then moved into the primary Fos antibody (Santa Cruz Biotechnology rabbit polyclonal anti-Fos K-25, sc-253, 1:10,000) in 10% Normal Goat Serum in PBT and incubated for 21 hours at 4°C. The K-25 Fos antibody has been extensively used in zebra finches (e.g [29]), and validated in songbirds [30]. Sections were then rinsed three times in PBT and incubated in biotinylated goat anti-rabbit secondary antibody (diluted 1:250 in PBT; Vector Laboratories) for 1 hour at room temperature. After another three rinses in PBT, sections were then incubated in avidin-biotin horseradish-peroxidase complex (1:400; Vector Laboratories) in PBT for 1 hour at room temperature. Following four rinses in PBT, one rinse in phosphate buffer, and a brief rinse in 0.1M sodium acetate, sections were visualised with 0.04% nickel-intensified diaminobenzidine (Sigma) solution for 210 s at room temperature to develop Fos immunoreactivity and then rinsed five times with phosphate buffer to terminate the reaction.

Immediately after Fos visualisation, we double-labelled each series to visualise tyrosine hydroxylase, vasotocin, or mesotocin. Tyrosine hydroxylase is an enzyme catalysing the rate-limiting step in dopamine synthesis and is used as a marker for dopaminergic neurones in vertebrate neuroanatomy (e.g. [6]). Briefly, tissue series were rinsed three times in PBT, once in phosphate buffer, and incubated in 0.3% H₂O₂ for 15 min. After another three PBT rinses, tissue series were incubated in blocking serum (tyrosine hydroxylase: 10% Normal Horse Serum, Vector; vasotocin and mesotocin: 3% Normal Goat Serum, Vector) in PBT for 60 min at room temperature. Sections were then transferred into PBT containing the appropriate blocking serum and primary antibody (tyrosine hydroxylase: mouse monoclonal; Millipore, MAB5280, 1:1000; vasotocin: rabbit polyclonal; a gift of Dr David A. Gray, University of the Witwatersrand, Johannesburg, South Africa, 1:10,000) and incubated for 60 h at 4°C. Sections destined for mesotocin double labelling were incubated in primary antibody (mesotocin: rabbit

polyclonal; Immunostar, 20068, 1:5000) for 87 hours at 4°C. All primary antibodies included in this study have been previously used and validated in birds (tyrosine hydroxylase: [e.g. 31], vasotocin: [32,33], mesotocin [34]). The vasotocin antibody exhibits no cross-reactivity with oxytocin, mesotocin and angiotensin (see [33]) and the mesotocin antibody exhibits no cross-reactivity with vasotocin (see [34]). The specificity of the TH antibody has been extensively addressed by the manufacturer (see manufacturer's data sheet) and has wide vertebrate species cross-reactivity including avian species. We also assessed antibody specificity by performing a series of immunohistochemical tests that did not result in any staining and these included complete omission of the primary or secondary antibodies. The distribution of cytoplasmic immunostaining in the zebra finch brain for each primary antibody was as expected when compared to the distribution of other published studies using different primary antibodies (tyrosine hydroxylase: [35]; mesotocin and vasotocin: [28]) and also for the known expression of each peptide mRNA. In addition, a dilution series of each primary antibody, when determining the optimum concentration of primary antibody to use, resulted in a correlated reduction in staining intensity. After three further rinses in PBT, tissue was incubated in a solution containing biotinylated secondary antibody (tyrosine hydroxylase: horse anti-mouse, 1:100, Vector; vasotocin and mesotocin: goat anti-rabbit, 1:100, Vector Laboratories) in PBT for 60 minutes at room temperature. After three rinses in PBT, sections were then incubated in avidin-biotin horseradish-peroxidase complex (1:50; Vector Laboratories) in PBT for 60 min at room temperature. After a final four rinses in PBT and a single rinse in phosphate buffer, the second label was visualised by incubating tissue in diaminobenzidine at room temperature for different periods of time depending on the tissue series (tyrosine hydroxylase: 110 s; vasotocin: 225 s; mesotocin: 140 s). Tissue was rinsed five times in phosphate buffer to terminate the diaminobenzidine reaction. This labelling procedure produced an intensely dark, black Fos labelled nuclei in neurones and a light brown cytoplasmic staining of neurones producing tyrosine hydroxylase, vasotocin, or mesotocin. After double-labelling, all tissue sections were mounted in series on to 0.5%

gelatine-subbed microscope slides (Thermo), serially dehydrated through alcohol (70 to 99%), cleared in xylene, and cover-slipped with Pertex mountant (CellPath).

Quantification of Fos immunoreactivity

We quantified Fos immunoreactivity in neuronal populations characterised by their production of vasotocin, mesotocin, or tyrosine hydroxylase. We located each neuronal population with reference to full-section architecture [36] and, more specifically, visualisation of vasotocin, mesotocin, and tyrosine hydroxylase. In both vasotocin- and mesotocin-labelled tissue, we sampled vasotocinergetic and mesotocinergetic populations in BSTmd in three adjacent sections and BSTmv in two adjacent sections in each brain. We were unable to sample Fos co-localisation in vasotocinergetic and mesotocinergetic neuronal populations in the supraoptic or paraventricular nuclei as vasotocin and mesotocin immunoreactivity in neurones in both of these regions was too intense to decipher whether Fos immunoreactivity was present in the nuclei of these neurones. In tyrosine hydroxylase-labelled tissue, we sampled tyrosine hydroxylase-immunoreactive (dopaminergic) populations in the ventral tegmental area in three adjacent sections and central gray in four adjacent sections in each brain.

In each neuronal population, we counted the number of neurones producing vasotocin, mesotocin, or tyrosine hydroxylase and the number of double-labelled (vasotocin+Fos, mesotocin+Fos, or tyrosine hydroxylase+Fos) neurones. Although tyrosine hydroxylase+Fos neurones could be counted in the ventral tegmental area visually using the microscope, single-labelled tyrosine hydroxylase-immunoreactive neuronal populations were too extensive to be quantified using this method. To count these neurones, we took photomicrographs of all ventral tegmental area sections using a 20x objective lens and counted the tyrosine hydroxylase-immunoreactive neurones using ImageJ software (version 1.45, NIH, Bethesda, MD, USA). All neurone counts were made in both hemispheres. To account for

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differences in vasotocinergic, mesotocinergic, and dopaminergic neuronal population sizes between sections and birds, we divided the total number of double-labelled cells by the total number of vasotocinergic, mesotocinergic, or dopaminergic neurones, respectively, in a given brain to quantify Fos immunoreactivity as the percentage of a neuronal population immunoreactive for Fos.

Statistical analysis

We used PASW software (version 19.00, SPSS Inc., Chicago, IL, USA) for all of our statistical analyses. We compared the amount of non-nesting behaviours exhibited by birds using GLMs with independent variables including sex on two levels (male and female) and treatment on two levels (nest building and control). We compared the number of song bouts and time spent singing between male control and nest-building birds using Mann-Whitney U test because this data did not conform to the assumptions of parametric statistical analysis. Similarly, we compared nest-building behaviours between nest-building male and female birds using Mann-Whitney U tests. Finally, we compared Fos immunoreactivity in each neuronal population using GLMs with independent variables including sex on two levels (male and female) and treatment on two levels (nest building and control), as above for non-nesting behaviour.

To investigate whether nest-building behaviour explained individual variation in Fos immunoreactivity, we used multiple linear regression including neuronal activity as a dependent variable and all recorded behaviours in nest-building birds as independent predictors. We ran regression models separately for each sex and each vasotocinergic, mesotocinergic, and dopaminergic neurone population sampled using

a stepwise reduction procedure to identify behaviours that significantly explained individual differences in Fos immunoreactivity in these populations.

Results

Behavioural analysis

Whereas control and nest-building birds did not differ in how often they hopped, preened, scratched, drank, and allopreened (all $p > 0.05$), control birds fed significantly more often than nest-building birds ($F_{1,26} = 3.494$, $p < 0.001$; control: 131.64 ± 13.38 feeds; nest-building: 32.63 ± 12.52 feeds). Control male birds also sang more often ($U = 9.0$, $p = 0.027$; control: 14 ± 5.7 song bouts, nest-building: 2.8 ± 0.7 song bouts) and for longer ($U = 9.0$; $p = 0.028$; control: 50.7 ± 21.6 s, nest-building: 12.6 ± 4.9 s) than nest-building males. Overall, control birds spent time feeding and, in the case of males, singing, when nest-building opportunities were not available.

Nest-building males picked up ($U = 64.0$, $p = 0.001$; male: 143.3 ± 36.5 pick ups, female: 8.8 ± 4.7 pick ups) and deposited ($U = 64.0$, $p < 0.001$; male: 68.8 ± 10.5 deposits, female: 0.1 ± 0.1 deposits) nest material significantly more often than nest-building females. Male and female nest-building finches did not differ in the number of times they tucked nest material into the nest structure ($U = 48.0$, $p = 0.093$). Nest-building male finches visited the nest cup more often ($U = 64.0$, $p = 0.001$; male: 79.1 ± 10.8 visits, female: 16.0 ± 2.9 visits) than nest-building females, however, male and female nest-building finches did not differ in the total amount of time they spent in the nest ($U = 36.5$, $p = 0.473$), suggesting females made fewer, but longer trips to the nest cup than their mates.

Vasotocinergic neuronal populations

Overall, Fos immunoreactivity in vasotocinergic neurone populations in BSTmd or BSTmv did not differ between nest-building birds and control birds (BSTmd: $F_{1,26} = 0.396$, $p = 0.535$; BSTmv: $F_{1,25} = 0.001$, $p = 0.978$).

Among nest-building males, Fos immunoreactivity in vasotocinergic neurones in BSTmd increased the more time a male spent together with his mate in the nest cup ($\beta = 0.837$; $t = 3.748$; $F_{1,6} = 14.048$; $p = 0.010$; Figure 1). Additionally, Fos immunoreactivity in vasotocinergic neurones in BSTmv increased the more times males picked up pieces of nest material ($\beta = 0.784$; $t = 3.097$; $F_{1,6} = 9.590$; $p = 0.021$; Figure 1). In the female mates of nest-building males, none of the behaviours we measured significantly explained the individual variation in Fos immunoreactivity in vasotocinergic populations in either BSTmd or BSTmv.

Mesotocinergic neuronal populations

Fos immunoreactivity in mesotocinergic neurones in BSTmd, but not in BSTmv, tended to be greater in the nest-building birds than it was in controls (BSTmd: $F_{1,26} = 4.160$, $p = 0.052$; BSTmv: $F_{1,25} = 0.612$; $p = 0.441$; Figure 2).

None of the behaviours that we measured significantly explained individual variation in Fos immunoreactivity in mesotocinergic neurones in either BSTmd or BSTmv in nest-building males or females.

Dopaminergic neuronal populations

Overall, Fos immunoreactivity in dopaminergic neurones in both the ventral tegmental area and central gray did not differ between the nest-building and control birds (ventral tegmental area: $F_{1,26} = 0.488$; $p = 0.491$; central gray: $F_{1,26} = 2.880$; $p = 0.102$).

Among nest-building males, Fos immunoreactivity increased in dopaminergic neurones in the central gray the more time a male spent with his mate in the nest cup ($\beta = 0.921$; $t = 5.793$; $F_{1,6} = 33.564$; $p = 0.001$; Figure 3). Additionally, Fos immunoreactivity in dopaminergic neurones in the ventral tegmental area decreased the more males tucked nest material into the nest ($\beta = -0.719$; $t = -2.531$; $F_{1,6} = 6.405$; $p = 0.045$).

In females paired with nest-building males, Fos immunoreactivity in dopaminergic neurones in the ventral tegmental area decreased the more a female fed ($\beta = -0.816$; $t = -3.453$; $F_{1,26} = 11.923$; $p = 0.014$). None of the behaviours that we measured significantly explained individual variation in Fos immunoreactivity in dopaminergic neurones in the central gray of female nesting finches.

Discussion

Vasotocinergic and mesotocinergic neuronal populations

Nonapeptide hormones and parental behaviour

The demonstration that vasotocinergic and mesotocinergic neuronal populations are active during nest building in zebra finches suggests that in addition to nonapeptide hormones acting in the brain to regulate pair formation [37], they may also be involved in nest building. In conjunction with previous studies demonstrating a role for nonapeptide signalling within the brain in parental behaviour in mammals [38] and fish [39], it appears that a role for nonapeptide hormone systems in parental behaviour may be evolutionarily conserved across vertebrate taxa. As recently emphasised by Kelly and Goodson [17], however, correlational studies demonstrating a relationship between neuronal activity in nonapeptidergic neuronal populations and social behaviours require complementary studies in which neuronal activity in these cells is manipulated within specific cell groups to establish a functional

connection between brain and behaviour. It should also be noted that because we used a relatively limited sample size and that Fos immunohistochemistry suffers from a lack of temporal specificity in relation to specific patterns of neuronal activity, the lack of a correlation between Fos immunoreactivity in a given neuronal population and a specific behaviour should not be used as evidence to preclude the involvement of that brain region in the behaviour of interest because some brain-behaviour relationships may not be detected using Fos immunohistochemistry or a smaller sample size.

Medial bed nucleus of the stria terminalis, dorsal subdivision (BSTmd)

In the present study, we found that the previously-reported increase in BSTmd Fos immunoreactivity in nest-building finches compared to non-building controls [9] may occur specifically within mesotocinergic neurones. Since this increase in immunoreactivity did not correlate with any nest-building behaviour, it may be related to nest possession or perception of the nest rather than to nest-building behaviour per se, consistent with the findings of Hall et al. [9]. This possibility requires explicit testing. The limited power in this group difference reported in Hall et al. [9] and here suggests that this group difference may be better tested with a larger sample size in the future.

Within BSTmd, Fos immunoreactivity in vasotocinergic neurones also increased in male nest-building finches the more time he spent together with his mate in their nest, a result that appears at odds with the absence of a relationship between Fos immunoreactivity in vasotocinergic neurones in BSTm and the time spent in the nest in zebra finches [28]. This discrepancy may be explained in part by differences in quantifying BSTmd and BSTmv Fos immunoreactivity separately or together (see Hall et al. [9]) and the behaviours measured. Whereas Klatt and Goodson [28] and Hall et al. [9] measured the amount of time individual birds spent within the nest, here we measured the amount of time the pair of finches spent together in the nest. This finding might be particularly important because vasotocinergic

neurons in BSTm appear to be involved in eliciting affiliative responses to mates [40]. These results support the hypothesis that vasotocinergic neuronal populations in BSTm of male birds may be involved in affiliative behaviour [17], including social interactions within the nest during nest building, although further studies are necessary to test this possibility.

Medial bed nucleus of the stria terminalis, ventral subdivision (BSTmv)

Although Fos immunoreactivity in the BSTmv was unrelated to nest-building behaviour in male finches in a previous study [9], here we found that Fos immunoreactivity in vasotocinergic neurons increased in BSTmv the more a nest-building male finch picked up nest material. This difference may be explained if the relationship between neuronal activity and picking up nest material is specific to vasotocinergic neurons in this region. The earlier result [9] may have been due to a masking of the total Fos immunoreactivity in other neuronal populations located within BSTmv. Functionally, vasotocinergic neurons in BSTmv of zebra finches may be involved in picking up nest material. Because antagonising vasotocin signalling in the brain did not affect nest material collection behaviour by male nest-building zebra finches [28], it is plausible that vasotocinergic neurons in BSTmv influence nest material collection through their neuronal activity. It should be noticed that the number of birds contributing to this relationship is limited and would benefit from replication using large sample sizes or manipulations to test for a functional relationship between BSTmv and nest material collection.

The lack of a relationship between Fos immunoreactivity in either vasotocinergic or mesotocinergic neuronal populations and the time a female spent in the nest suggests that other neuronal populations located in BSTmv intermingled with the nonapeptidergic populations sampled here, such as the population of neurons expressing receptors for vasoactive intestinal peptide [41], may increase their activity the more time a female finch spends in the nest. This vasoactive intestinal peptide-

sensitive neuronal population may be particularly interesting in the context of nest building because vasoactive intestinal peptide signalling regulates prolactin release which is involved in maternal behaviour including incubation in birds [42]. The involvement of another neuronal population aside from vasotocinergic and mesotocinergic neurones in the relationship between neuronal activity in BSTmv and time spent in the nest also explains why central infusions of pharmacological antagonists that impair vasotocin and mesotocin signalling did not affect the time female zebra finches spent within the nest [28].

Dopaminergic neuronal populations

Ventral tegmental area

Fos immunoreactivity in dopaminergic neurones within the ventral tegmental area was not correlated with nest material collection by male finches, so it appears that this dopaminergic neuronal population does not play a role in collecting nest material. The decrease in Fos immunoreactivity in ventral tegmental area dopaminergic neurones the more nest-building male finches tucked material into the nest structure may instead suggest that tucking nest material into the nest structure is unrewarding or that the dopaminergic neurone population in the ventral tegmental area inhibits tucking behaviour in male finches. Pharmacological manipulations could be used to inhibit neuronal activity in ventral tegmental area dopaminergic neurones in order to distinguish between these two possibilities.

Interestingly, Banerjee et al. [43] found that Fos immunoreactivity increased in dopaminergic neurones in the ventral tegmental area in recently paired male and female zebra finches, who were also observed to spend more time in the nest together than controls. Here, we found no relationship between time spent in the nest together with Fos immunoreactivity in dopaminergic neurons in the ventral

tegmental area, however, we also waited a minimum of one week following pairing to record nest-building behaviour, suggesting this increased neuronal activity may be specific to recently-paired birds.

Finally, in female nest-building finches, we found that Fos immunoreactivity in dopaminergic neurons in the ventral tegmental area decreased the more often these females fed. This negative relationship contrasts with the well-known relationship dopamine has in the ventral tegmental area mediating food rewards (e.g. [44]). It should be noted that this negative relationship only appeared in our nest-building female finches and studies dedicated to identifying neural substrates of feeding in birds are required to begin elucidating the biological significance of this result.

Central gray

The increase in Fos immunoreactivity in central gray dopaminergic neurones in male nest-building finches the more time he spent in the nest with his partner supports the proposal that dopaminergic neurones in the central gray may play a role in social communication [3] between a male finch and his female partner. Consistent with the central gray playing a role in pair interaction during nest building specifically, Banerjee et al. [43] found that Fos immunoreactivity increased in dopaminergic neurons in the central gray of male finches that had recently been paired with a female and spent more time in the nest compared to controls. It is possible that this social communication takes the form of “duet-like” vocalisations that appear to be performed during nest building [45] but, as yet, we have no data to confirm this possibility.

Together, these data provide the first evidence that vasotocinergic, mesotocinergic, and dopaminergic neuronal populations in the social behaviour network and dopaminergic reward system are active when birds are nest building. These brain-behaviour relationships suggest that nest-building behaviour can be

classified as a social behaviour regulated by the social behaviour network and dopaminergic reward system and provide a robust neuroendocrinological foundation for future studies on the neurobiology of nest-building behaviour. Furthermore, these data support the suggestion that nonapeptidergic systems in the brain play an evolutionarily conserved role in controlling parental behaviour in vertebrates, including nest building in birds.

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Figure Legends

Figure 1. (A) Photomicrograph of medial bed nucleus of the stria terminalis (BST) immunocytochemically labelled for arginine vasotocin and Fos with dotted lines indicating the boundaries of vasotocinergic neuronal populations sampled in this study. (B) Correlation between the time a pair of nest-building zebra finches spent together in the nest and the percentage of arginine vasotocin immunoreactive (AVT-ir) neurones in the BST, dorsal subdivision (BSTmd) immunoreactive for Fos in males. (C) Correlation between the number of times male nest-building zebra finches picked up pieces of nest material and the percentage of vasotocinergic neurones in the BST, ventral subdivision (BSTmv) immunoreactive for Fos in males. AC = anterior commissure.

Figure 2. (A) Fos immunoreactivity in mesotocin-immunoreactive (MT-ir) neurones in the medial bed nucleus of the stria terminalis, dorsal subdivision (BSTmd) in adult control and nest-building zebra finches. Bars represent mean percentage of MT-ir neurones immunoreactive for Fos in BSTmd in female (white bars) and male (black bars) zebra finches of pairs in which the male was or was not constructing a nest \pm SEM. The n value for each group is printed within each bar on the graph. (B) A micrograph of neurones in the BSTmd immunoreactive for MT (cytoplasmic brown stain) and Fos (black nuclear stain) in a male nest-building zebra finch. Arrows indicate examples of double-labelled cells. Scale bar = 20 μ m.

Figure 3. (A) Correlation between the time a pair of nest-building zebra finches spent together in the nest cup and Fos immunoreactivity in tyrosine hydroxylase-immunoreactive (TH-ir) neurones in the central gray (CG) of male zebra finches. (B) A photomicrograph of neurones labelled for TH (cytoplasmic brown stain) and Fos (black nuclear stain) immunoreactivity. Arrows indicate neurones containing both labels. Scale bar = 20 μ m.



